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RAPID COUNTING OF NEMATODA IN SALMON BY PEPTIC DIGESTION

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Contribution No. 2 to research conducted with the approval of the United States Section of the International North Pacific Fisheries Commission. The Commission, established in 1953 by the International Convention for the High Seas Fisheries of the North Pacific Ocean, coordinates the research of the member nations: Japan, Canada, and the United States. The resulting investigations provide data to the Commission for use in carrying out its duties in connection with fishery conservation problems in the North Pacific Ocean. Publication of this scientific report has been approved by the United States Section of the Commission.

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ABSTRACT

The nematode parasite Anisakis sp. can be recovered, relatively unaltered, from chum salmon musculature by high temperature (52° ± 2° C.) peptic digestion of the flesh. The procedure, which is presented in detail, appears to be more thorough in isolating the parasite than dissection of the flesh and manual isolation of the worms. In addition, the procedure is rapid, comparatively simple, economical, and suitable for the examination of large numbers of samples.

The population of Anisakis sp. larvae in chum salmon appears to be concentrated in the ventral section of the fish, that is, below the lateral line, which suggests that only the ventral quarters of the fish need to be examined for estimating infection intensity.

RAPID COUNTING OF NEMATODA IN SALMON BY PEPTIC DIGESTION

Introduction

A major problem in the management of the international North Pacific salmon fishery is the resolution of a line or corridor in the North Pacific Ocean which equitably divides high seas stocks of salmon according to their continental origin. One of the techniques for the characterization of the salmon stocks employs the use of naturally occurring parasites as indexes of racial likeness.

Among the more promising of the "tracer parasites" is the larval nematode Anisakis sp. Criteria for specific identification of Anisakis larvae are not well established. The species considered in this study may be one of several reputed species from fish-eating mammals. The larvae in salmon appear to be morphologically identical with those in herring. Furthermore, the ecological background of herring and salmon have much in common to support a hypothesis of identity between these larval parasites. Bishop and Margolis (1955) considered the Anisakis larvae in herring (Clupea pallasii) from the coast of British Columbia to be the intermediate stage of Anisakis simplex (Rudolphi, 1809), a stomach parasite of whales and seals. Whatever the taxonomic status of these forms, there is no evidence to suggest that more than one species is represented in salmon.

The worms are colorless, translucent organisms which usually occur, to a greater or lesser degree, tightly coiled in lenticular cysts on serosal surfaces of visceral organs and in the somatic musculature of the salmon, within and between the myotomes. The difficulty of detection, recovery, and enumeration of these parasites in salmon musculature by the routine method of dissection and direct observation is a deterrent to a satisfactory rate of progress for thorough quantitative analysis of the infection phenomenon. Therefore, an investigation was undertaken to explore and develop other suitable methods of isolation and enumeration of the larval worms.

Various methods of chemical and enzymatic digestion of the host musculature were studied. Of these, only peptic digestion at relatively high temperatures proved efficient and economically suitable for use with large numbers of samples.

Procedure

The fish are filleted and skinned. Each fillet is weighed to the nearest gram and then broken into several smaller pieces to increase initial surface area. The material from an individual fillet is placed in a digestion vessel (a beaker or jar of approximately 4 liters' capacity). Five milliliters per gram of flesh of a 0.25-percent pepsin solution (2.5 grams of granular pepsin, 1:10,000 made to 1 liter with 1-percent HCl) are added to the vessel. The mixture is stirred gently, and the pH is adjusted to 1.2 with concentrated HCl.

The digestion vessel is placed in a constant-temperature water bath or air oven where the temperature of the digestion mixture is raised to and maintained at the temperature optimum for the most rapid digestion (52° ± 2° C.). The digestion is permitted to continue for 15 minutes after the optimum temperature has been attained. During the heating and holding periods, the mixture is stirred mechanically at a moderately high speed using a glass stirring rod.

Following the holding period, the mixture is permitted to stand at room temperature for 5 minutes without agitation. During this period, the peptonized tissue forms a layer at the top of the solution, and the worms settle to the bottom. The layer of peptonized material is decanted. The remaining liquid is agitated to suspend the worms and then filtered through a standard 80-mesh grading sieve. The residue on the screen is washed several times with cold water and then transferred to a large Petri dish to which is added a few milliliters of 10 percent formaldehyde.

The formaldehyde further denatures the proteinaceous components and causes the worms to appear opaque and whitish in color, suitable for rapid enumeration against a dark background.

Evaluation of the Procedure

The relative efficiencies of recovery of the parasites by the dissection method and by the peptic digestion procedure first were evaluated. Thirty-nine chum salmon (Oncorhynchus keta), obtained from various areas of the North Pacific Ocean, were used. The right sides of the fish were treated by dissection and manual isolation of the worms; the left sides by peptic digestion. The results of the study are presented in table 1.

The data in table 1 were analyzed statistically with the "t" test for the difference of mean values (Johnson, 1950). The population of worms isolated by dissection was found to be significantly less ($t = 2.03$, $N = 39$, significant at $P = 0.25$) than that determined by the digestion procedure.

The possibility existed that the difference might have been due to dissimilarities either in efficiency of recovery of the two procedures or in the distribution of the worms between the left and right sides of the fish. To determine which of these possibilities was most probable, a second study was initiated. In this study, 15 chum salmon were used. The parasites in both the right and left sides of each fish were first dissected and removed for enumeration. The dissected material then was treated by peptic digestion, and the residual entire worms in the flesh were isolated and counted. The data, which are independent of these in table 1, are presented in table 2.

A statistical analysis of the data, again using the "t" test for the difference of mean values, demonstrated that the number of parasites determined by the dissection method alone was significantly less ($t = 3.26$, $N = 15$, significant at $P = 0.01$) than the total number counted, i.e., the number determined by dissection plus the additional number recovered by digestion of the dissected material.

The mean difference between the total number counted and the number obtained by the dissection of the fish was taken as an

Table 1.--Anisakis sp. count by dissection and digestion.

Sample No.	Count by dissection	Count by digestion	Sample No.	Count by dissection	Count by digestion
1	7	9	21	11	22
2	7	44	22	33	34
3	8	17	23	12	14
4	14	10	24	11	12
5	19	9	25	23	13
6	40	30	26	25	30
7	43	79	27	27	22
8	25	62	28	10	7
9	77	54	29	9	15
10	26	50	30	41	25
11	18	16	31	7	14
12	25	21	32	36	53
13	9	12	33	12	14
14	34	70	34	19	14
15	25	26	35	31	24
16	9	8	36	13	22
17	22	38	37	14	20
18	10	18	38	19	15
19	5	3	39	13	11
20	21	30			
			TOTAL	810	987
			MEAN	20.76	25.30

Table 2.--Anisakis sp. count by dissection, digestion of dissected material, and total count.

(Summation of the two independent determinations)

Sample No.	Count by dissection	Count by digestion of dissected portion	Total count
1	18	0	18
2	25	2	27
3	51	5	56
4	16	3	19
5	57	2	59
6	100	17	117
7	67	6	73
8	6	2	8
9	6	1	7
10	34	4	38
11	22	6	28
12	9	1	10
13	3	3	6
14	0	0	0
15	11	1	12
TOTAL	425	53	478
MEAN	28.33	3.53	31.86

approximation of a correction factor for data obtained by dissection. The correction factor was added to each of the individual dissection counts presented in table 1, and an analysis of the corrected dissection data and the digestion data in table 1 was carried out. This analysis (table 3) demonstrated that a significant difference did not exist ($t = 0.50$, $N = 39$, not significant at $P = 0.50$) which indicated that the significant difference obtained with the uncorrected data in table 1 was due to the difference in efficiency of recovery of the worms by the two methods rather than to differences in the distribution of the worms between the two sides of the fish.

It should be realized that the correction factor used in this analysis was merely a first approximation. A true correction factor probably would be a function of the number determined by the dissection procedure. The determination of an accurate factor would require more data than obtained in the present study.

Preliminary observations indicated that the population of Anisakis sp. larvae was concentrated in the musculature of the ventral section of the fish. Thirty fillets of chum salmon, each obtained from the right side of a fish, were divided along the lateral lines into dorsal and ventral sections. The sections were digested separately, and the number of worms in each of the sections was determined. The data, which are presented in table 4, demonstrate the preponderance of worms in the hypaxial musculature.

Table 3.--Correction of Anisakis sp. count of table 1 using the correction factor as described in text.

Sample No.	Corrected dissection count	Count by digestion	Sample No.	Corrected dissection count	Count by digestion
1	10.5	9	21	14.5	22
2	10.5	44	22	36.5	34
3	11.5	17	23	15.5	14
4	17.5	10	24	14.5	12
5	22.5	9	25	26.5	13
6	43.5	30	26	28.5	30
7	46.5	79	27	30.5	22
8	28.5	62	28	13.5	7
9	80.5	54	29	12.5	15
10	29.5	50	30	44.5	25
11	21.5	16	31	10.5	14
12	28.5	21	32	39.5	53
13	12.5	12	33	15.5	14
14	37.5	70	34	22.5	14
15	28.5	26	35	34.5	24
16	12.5	8	36	16.5	22
17	25.5	38	37	17.5	20
18	13.5	18	38	22.5	15
19	8.5	3	39	16.5	11
20	24.5	30	TOTAL	946.5	987
			MEAN	24.26	25.30

Discussion

The application of enzymatic proteolysis to the recovery of tissue-bound parasites has found considerable use in parasitology (Gustavson, 1953; Hoffman, 1955; among others). The successful use of enzymatic digestion in the case of nematodes undoubtedly depends upon the resistance of the cuticle to the proteolytic attack of the specific enzyme used. The resistance is due to the collagenous nature of the cuticle as demonstrated by Chitwood (1936) and more recently by Bird (1957).

In all cases of which the authors are aware, the enzymatic digestion techniques have been pointed toward recovery of living parasites for subsequent use in morphological, physiological, or infectivity studies. Under these conditions, enzymatic digestion of the investing tissue must be carefully controlled, lest the physical conditions of the system, notably temperature and hydrogen-ion concentrations, exceed the tolerance of the parasite in question. The enzymes pepsin and trypsin, for example, most

Table 4.--Anisakis sp. count by digestion method on dorsal and ventral portions of the sides of fish.

Sample No.	Total count	Dorsal count	Ventral count	Sample No.	Total count	Dorsal count	Ventral count
1	50	0	50	16	13	0	13
2	16	1	15	17	30	0	30
3	21	1	20	18	22	2	20
4	12	3	9	19	7	1	6
5	70	2	68	20	15	0	15
6	26	1	25	21	25	0	25
7	8	2	6	22	14	1	13
8	38	3	35	23	53	0	53
9	18	1	17	24	14	0	14
10	3	0	3	25	14	0	14
11	30	2	28	26	24	0	24
12	22	2	20	27	22	0	22
13	34	2	32	28	20	0	20
14	14	1	13	29	15	0	15
15	12	0	12	30	11	1	10
				TOTAL	673	26	647
				MEAN	22.43	0.86	21.56

typically are used in solutions maintained at temperatures not exceeding 37° or, at a maximum, 39° C., which are temperatures that are obviously optimal, or nearly so, for all animal parasites potentially infective, at the stage of recovery, for homothermous hosts. In the event that it is known or assumed, a priori, that the parasite is infective for a poikilotherm, the temperature of the digest solution is changed accordingly.

The present investigation concerns the use of peptic digestion in extensive quantitative studies which are not concerned with the recovery of viable parasites. Under the conditions of the present investigation, certain advantages are evident in what may be termed high-temperature peptic digestion. Economy was important, and therefore the use of minimal concentrations of enzyme was desired. In addition, time for quantification was considered of utmost importance.

For enzyme reactions, time and temperature are interdependent variables. The actual temperature at which an enzyme reaction proceeds at a maximum rate depends on many factors such as the purity and concentration of the enzyme and substrate, the presence of activators or inhibitors, and the method of following the reaction. As has been well established (Baldwin, 1953; Neilands and Stumpf, 1955), the so-called optimum temperature is a function of the time chosen for rate measurement. For any given set of experimental conditions, it is possible to determine the temperature at which the greatest amount of chemical change is catalyzed by an enzyme under that particular set of conditions. Owing to the susceptibility of enzymes to thermal inactivation, at temperatures lower than the optimum the enzyme will be relatively more stable and the reaction will proceed more slowly but will continue longer, resulting in greater concentrations of end products. At temperatures greater than the optimum, the reaction will proceed more rapidly, but the catalytic properties of the enzyme will be more rapidly destroyed (Baldwin, 1953).

A series of experiments were carried out to determine the optimum temperature for peptonization which would require a minimum of time and a minimum of enzyme concentration. The results of these experiments demonstrated that with 5 milliliters of 0.25 percent pepsin solution per gram of flesh, 15 to 20 minutes at 50° - 54° C. was sufficient for peptonization of the flesh and complete separation of worms from the musculature. At successive temperatures lower than 50° C., the time for complete peptonization was lengthened so that it prohibited accumulating mass data; at successive temperatures greater than 54° C., thermal inactivation of the enzyme interfered with the digestion.

In addition to the simplicity and rapidity of the method, the high-temperature digestion procedure is efficient in that the worms are easily recognized, and recovery of all the larvae is facilitated. Furthermore, personal error and fatigue are not likely to affect the results as much as in the case of the more tedious procedure of dissection and manual isolation.

The procedure is economical in that the necessary equipment is simple and inexpensive. The principal cost is that of pepsin, which was found to average slightly less than $0.0005 per gram of flesh digested. In the present investigation, the fish were small, and the average weight of the fillets was 400 grams; the cost of pepsin per fillet was $0.20, or $0.40 per fish. If only the ventral portions of the fillets had been used for the enumeration studies, the cost per fish would have been approximately $0.18.

The procedure also offers the advantage that it lends itself to multiple determinations. During the course of the investigation, it was found that 2 people, working with a total of 5 digestion vessels, could prepare the necessary solutions and treat and enumerate the parasites in 50 fillets during a normal working day. Thus, a single operator can examine four times as many fish in a unit of time as with the dissection method.

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